NeoBiota 84: 247–266 (2023)
doi: 10.3897/neobiota.84.90843
https://neobiota.pensoft.net

RESEARCH ARTICLE

A peer-reviewed open-access journal
NeoBiota
Advancing research on alien species and biological invasions

Duplex real-time PCR assay for the simultaneous detection of *Ophiostoma novo-ulmi* and *Geosmithia* spp. in elm wood and insect vectors

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Academic editor: Christophe Orazio | Received 25 July 2022 | Accepted 13 February 2023 | Published 18 May 2023

Citation: Pepori AL, Luchi N, Pecori F, Santini A (2023) Duplex real-time PCR assay for the simultaneous detection of *Ophiostoma novo-ulmi* and *Geosmithia* spp. in elm wood and insect vectors. In: Jactel H, Orazio C, Robinet C, Douma JC, Santini A, Battisti A, Branco M, Seehausen L, Kenis M (Eds) Conceptual and technical innovations to better manage invasions of alien pests and pathogens in forests. NeoBiota 84: 247–266. https://doi.org/10.3897/neobiota.84.90843

Abstract

Dutch elm disease (DED) is a destructive tracheomycosis caused by *Ophiostoma novo-ulmi*, an ascomycete probably originating in East-Asia that is devastating natural elm populations throughout Europe, North America and Asia. The fungus is mainly spread by elm bark beetles that complete their life cycle between healthy and diseased elms. Recently, it has been highlighted that some fungi of the genus *Geosmithia*, which are similarly well associated with bark beetles, seem to also play a role in the DED pathosystem acting as mycoparasites of *O. novo-ulmi*. Although some relationship between the fungi is clear, the biological cycle of *Geosmithia* spp. within the DED cycle is still partly unclear, as is the role of *Geosmithia* spp. in association with the bark beetles. In this work, we tried to clarify these aspects by developing a qPCR duplex TaqMan assay to detect and quantify DNA of both fungi. The assay is extremely sensitive showing a limit of detection as low as 2 fg μl^{-1} for both fungi. We collected woody samples from healthy and infected elm trees throughout the beetle life cycle. All healthy elm samples were negative for both *Geosmithia* spp. and *O. novo-ulmi* DNA. *Geosmithia* spp. are never present in infected, but living trees, while they are present in frass of elm bark beetles (EBB – *Scolytus* spp.) and at each stage of the EBB life cycle in much higher quantities than *O. novo-ulmi*. This work provides a better understanding of the role and interactions occurring amongst the main players of the DED pathosystem.

Keywords

DNA quantification, duplex qPCR, Dutch Elm Disease, *Geosmithia* spp. life cycle, *Ophiostoma novo-ulmi*, *Scolytus multistriatus*

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Introduction

Dutch elm disease (DED) is a destructive tracheomycosis that has devastated natural elm populations throughout Europe, North America and Asia. The disease is caused by two subspecies of *Ophiostoma novo-ulmi* Brasier, i.e. ssp. *novo-ulmi* and ssp. *americana*, previously known as Eurasian (EAN) and North American (NAN) races, respectively (Brasier and Kirk 2001). These ascomycetes are responsible for the ongoing DED pandemic; since the 1970s, they have replaced the less aggressive *O. ulmi* (Buisman) Nannf. that caused the first DED pandemic at the beginning of the last century (Spierenburg 1921).

The fungus is mainly spread by species of elm bark beetles (Coleoptera, Curculionidae, Scolytinae) that complete their life cycle between healthy and diseased elms. Bark beetles belonging to the genus *Scolytus* Geoffroy are the main vectors of *O. ulmi* s.l. (Webber and Brasier 1984). Specifically, *S. scolytus* (F.) and *S. multistriatus* (Marsham), the large and small elm bark beetles (EBB), respectively, are the most common and important species spreading the pathogen worldwide (Webber and Kirby 1983; Webber and Brasier 1984; Webber and Gibbs 1989; Webber 1990, 2000; Faccoli 2001, 2004). The small EBB is the main vector in the Mediterranean area (Santini and Faccoli 2015). During spring, at the time of beetle flight, host plants are more prone to be infected and temperatures are favourable for fungal growth in plant tissue, enhancing the pathogen's aggressiveness (Santini and Faccoli 2015). Callow adults, carrying the *O. novo-ulmi* conidia, feed at the crotches of 1–2 years-old twigs of adult healthy elm trees to complete their sexual development, thus inoculating the pathogen. Once inoculated, the spores germinate into a growing mycelium and reach the xylem, where the fungus moves into the vessels (Webber and Brasier 1984), inducing the formation of tyloses and gels in the xylem vessels (Stipes and Campana 1981; Rioux et al. 1998; Ouellette et al. 2004a, b; Et-Touil et al. 2005) as a defence response. Later, the beetles move to dying elms to lay eggs in the inner bark of the trunks or branches, which provide the optimal environment for larval development (Rudinsky 1962) and fruiting of the pathogen (Webber and Brasier 1984). New contaminated beetles emerge from the bark to complete the cycle.

Recently, it has been highlighted that other organisms also play roles in the DED pathosystem (Pepori et al. 2018). Some fungi of the genus *Geosmithia*, a monophyletic morphogenus of anamorphic ascomycetes mainly associated with phloem-feeding bark beetles (Kolařík et al. 2004, 2005, 2007, 2008; Kubátová et al. 2004; Kolařík and Jankowiak 2013; McPherson et al. 2013; Jankowiak et al. 2014; Machingambi et al. 2014; Huang et al. 2019; Crous et al. 2022; Meshram et al. 2022), are consistently found in infected elms (Kolařík et al. 2004, 2005, 2007, 2008; Pepori et al. 2015; Huang et al. 2019; Strzałka et al. 2021; Crous et al. 2022).

Geosmithia spp., like *O. novo-ulmi*, are associated with elm bark beetles (Pepori et al. 2015, 2018) and can similarly be found in beetle larval galleries – thus sharing habitat with *O. novo-ulmi* – but the ecological niches of these fungi are different. A widespread horizontal gene transfer of the *cerato-ulmin* gene between *O. novo-ulmi* and *Geosmithia* species has been reported (Bettini et al. 2014).

Pepori et al. (2018) demonstrated the existence of a close and stable relationship, which can be classified as mycoparasitism by *Geosmithia* spp. towards *O. novo-ulmi*. There are still several gaps in defining the life cycle and lifestyle of elm-related *Geosmithia* species, especially when they cross and interact with the life cycle of DED fungi.

Previously, several methods of biocontrol of *O. novo-ulmi* have been investigated and have appeared promising under experimental conditions, although their practical application in the field has been limited (Webber and Gibbs 1984; Webber and Hedger 1986; Sutherland and Brasier 1995; Brasier 2000; Griffin 2000; Hintz et al. 2013; Ganley and Bulman 2016).

An accurate description of the life cycle and identification of the key factors that can enhance the attitude of *Geosmithia* spp. to act as effective biocontrol agents against *O. novo-ulmi* may be strategic in controlling the further spread of the disease.

In this study, a new, ad hoc duplex real-time PCR assay, based on TaqMan probe chemistry genus-specific for *Geosmithia* and species-specific for *O. novo-ulmi*, for the simultaneous quantification of both fungi from different matrices, was developed. Application of this molecular approach will fill the knowledge gaps related to the life cycle of *Geosmithia* spp. and will uncover the tripartite interactions amongst *O. novo-ulmi*, *Geosmithia* spp. and EBBs.

Materials and methods

Fungal strains

The duplex qPCR assay was validated using 12 isolates of *Geosmithia* spp. belonging to nine different species (*G. fassiatiae*, *G. flava*, *G. funiculosa*, *G. langdonii*, *G. lavendula*, *G. obscura*, *G. omnicola*, *G. pallida* and *G. putterillii*) and eight isolates of *Ophiostoma* from five species (*O. himal-ulmi*, *O. novo-ulmi* ssp. *novo-ulmi*, *O. novo-ulmi* ssp. *americana*, *O. quercus* and *O. ulmi*). Two ubiquitous species were also included as outgroups (Table 1). All fungal strains were obtained from the Institute for Sustainable Plant Protection – National Research Council (IPSP-CNR, Florence, Italy) collection (Table 1). Fungal isolates were grown on 300PT cellophane discs (Celsa, Varese, Italy) on 1.5% Malt Extract Agar (MEA; Difco Laboratories, Detroit, MI) in 90 mm Petri dishes and incubated in the dark at 20 °C. After 10 days, the mycelium was scraped from the surface of the cellophane and stored in 1.5 ml microfuge tubes (Sarstedt, Verona, Italy) at -20 °C. Fungal mycelium (ca. 100 mg fresh weight) was transferred into a 2-ml microfuge tube (Sarstedt) with two tungsten beads (3 mm) (Qiagen, Hilden, Germany) and ground with a Mixer Mill 300 (Qiagen) (2 min; 20 Hz). DNA extraction was performed using the E.Z.N.A. Plant DNA Mini Kit (Omega Bio-tek, Norcross, GA, USA), following the manufacturer's instructions. The concentration of extracted DNA was measured using a Nanodrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA).

Table 1. Fungal strains used in this study.

Species	Isolate Code	Host	Origin	Duplex qPCR[a] (*O. novo-ulmi*/ *Geosmithia* spp.)
Geosmithia fassiatiae	CCF3334	*Quercus pubescens*	Czech Republic	(-/+)
G. flava	MK1551	*Pteleobius vittatus* (on *Ulmus laevis*)	Czech Republic	(-/+)
G. funiculosa	IVV7	*U. minor*	Italy	(-/+)
G. funiculosa	CNR28	*U. minor*	Czech Republic	(-/+)
G. langdonii	MK1643	*Scolytus multistriatus* (on *U. laevis*)	Czech Republic	(-/+)
G. langdonii	MK1644	*Scolytus multistriatus* (on *U. laevis*)	Czech Republic	(-/+)
G. lavendula	CCF3394	*Chaetopyelius vestitus* (on *Pistacia terebinthus*)	Croatia	(-/+)
G. obscura	MK86	*Scolytus intricatus* (on *Quercus robur*)	Czech Republic	(-/+)
G. omnicola	CNR5	*U. minor*	Czech Republic	(-/+)
G. omnicola	CNR21	*U. minor*	Czech Republic	(-/+)
G. pallida	MK1622	*S. kirschii* (on *U. minor*)	Spain	(-/+)
G. putterillii	CCF3342	*Scolytus rugulosus* (on *Prunus* sp.)	Czech Republic	(-/+)
Ophiostoma himal-ulmi	CBS374.67	*U. wallichiana*	India	(-/-)
O. novo-ulmi ssp. *novo-ulmi*	CKT11	*Ulmus* sp.	Iran	(+/-)
O. novo-ulmi ssp. *novo-ulmi*	R64	*Ulmus* sp.	Romania	(+/-)
O. novo-ulmi ssp. *americana*	H172	*Ulmus* sp.	USA	(+/-)
O. novo-ulmi ssp. *americana*	H363	*Ulmus* sp.	Ireland	(+/-)
O. quercus	CBS722.95	*Quercus* sp.	Austria	(-/-)
O. ulmi	E2	*Ulmus* sp.	Netherlands	(-/-)
O. ulmi	R21	*Ulmus* sp.	Romania	(-/-)
Epiccoccum sp.	F15	*Q. suber*	Italy	(-/-)
Cladosporium sp.	F11	*Q. suber*	Italy	(-/-)

[a] + = positive qPCR amplification ; – = no amplification products.

Sampling on elm trees and bark beetle collections

Elm bark beetle (EBB) here means exclusively *Scolytus multistriatus* (Marsham), as it is the most common, active and effective DED vector in Italy and the only one found during sampling.

A total of 123 samples were collected from: i) wood of healthy elm trees; ii) dying elm trees showing DED symptoms (wood from newly-DED infected tissues, wood from old DED infections, living EBB larvae, living EBB pupae and wood frass from maternal and larval galleries); iii) EBB callow adults in flickering traps; and iv) adult females in galleries after oviposition (Table 2). All samples were collected in 1.5 ml microfuge tubes (Sarstedt), frozen in liquid nitrogen and immediately brought to the IPSP-CNR laboratory facilities to be stored in a -80 °C freezer before DNA extraction.

DNA extraction from woody samples and insects

Each woody sample (approx. 100 mg fresh weight from each collected tree and frass) and each insect sample (approx. 5.4 mg fresh weight –containing up to 4 larvae or pupae collected alive) was transferred into 2-ml microfuge tubes (Sarstedt), each containing two tungsten beads (Qiagen) and ground with a Mixer Mill 300 (Qiagen) (2 min; 20 Hz). DNA extraction was performed by using the E.Z.N.A. Plant DNA Minikit (Omega Bio-tek), according to the manufacturer's instructions.

Table 2. List of samples collected and tested in this study.

Source	N° of collected samples	Species	Sample	Geographic orgin (Lat., Long.)
Healthy trees	8	*Ulmus minor*	Wood	Florence, Italy (43.772402°N, 11.176578°E)
	6	*U. minor*	Wood	Sesto Fiorentino, Italy (43.817554°N, 11.188349°E)
New DED infection	7	*U. minor*	Wood	Siena, Italy (43.317361°N, 11.306896°E)
	4	*U. minor*	Wood	Castelnuovo Berardenga, Italy (43.341865°N, 11.519271°E)
	3	*U. minor*	Wood	Asciano, Italy (43.296617°N, 11.460314°E)
Old DED Infections	6	*U. minor*	Wood	Bagno a Ripoli, Italy (43.734871°N, 11.324844°E)
	4	*U. minor*	wood	Montelupo Fiorentino, Italy (43.720481°N, 10.988996°E)
	3	*U. minor*	Wood	Florence, Italy (43.811942°N, 11.240917°E)
	2	*U. minor*	Wood	Castagneto Carducci, Italy (43.194141°N, 10.567814°E)
	2	*U. minor*	Wood	Asciano, Italy (43.296617°N, 11.460314°E)
	2	*U. minor*	Wood	Poggibonsi, Italy (43.476425°N, 11.180486°E)
	2	*U. minor*	Wood	Castelnuovo di Val di Cecina, Italy (43.267503°N, 10.960795°E)
	1	*U. minor*	Wood	Asciano, Italy (43.296617°N, 11.460314°E)
	1	*U. minor* 'CEM187'	Wood	Bagno a Ripoli, Italy (43.734871°N, 11.324844°E)
	1	*U. minor* 'CEM370'	Wood	Bagno a Ripoli, Italy (43.734871°N, 11.324844°E)
	1	*U. minor*	Wood	Chiusdino, Italy (43.163653°N, 11.088422°E)
	1	*U. minor*	Wood	Castelnuovo Berardenga, Italy (43.341865°N, 11.519271°E)
Frass from EBB galleries	4	*U. minor*	Wood frass	Poggibonsi, Italy (43.476425°N, 11.180486°E)
	3	*U. minor*	Wood frass	Castelnuovo di Val di Cecina, Italy (43.267503°N, 10.960795°E)
	3	*U. minor*	Wood frass	Sesto Fiorentino, Italy (43.817554°N, 11.188349°E)
	2	*U. minor*	Wood frass	Chiusdino, Italy (43.163653°N, 11.088422°E)
EBB larvae	5	*Scolytus multistriatus*	Larvae	Montelupo fiorentino, Italy (43.720481°N, 10.988996°E)
	3	*S. multistriatus*	Larvae	Castelnuovo di Val di Cecina, Italy (43.267503°N, 10.960795°E)
	2	*S. multistriatus*	Larvae	Chiusdino, Italy (43.163653°N, 11.088422°E)
EBB pupae	4	*S. multistriatus*	Pupae	Castelnuovo di Val di Cecina, Italy (43.267503°N, 10.960795°E)
	2	*S. multistriatus*	Pupae	Montelupo fiorentino, Italy (43.720481°N, 10.988996°E)
	2	*S. multistriatus*	Pupae	Chiusdino, Italy (43.163653°N, 11.088422°E)
EBB in the galleries	5	*S. multistriatus*	Insect	Asciano, Italy (43.296617°N, 11.460314°E)
	2	*S. multistriatus*	Insect	Castagneto Carducci, Italy (43.194141°N, 10.567814°E)
	1	*S. multistriatus*	Insect	Florence, Italy (43.811942°N, 11.240917°E)
	1	*S. multistriatus*	Insect	Montelupo fiorentino, Italy (43.720481°N, 10.988996°E)
EBB callow adult	11	*S. multistriatus*	Insect	Sesto Fiorentino, Italy (43.817554°N, 11.188349°E)
	6	*S. multistriatus*	Insect	Montelupo fiorentino, Italy (43.720481°N, 10.988996°E)
	4	*S. multistriatus*	Insect	Florence, Italy (43.811942°N, 11.240917°E)
	4	*S. multistriatus*	Insect	Castagneto Carducci, Italy (43.194141°N, 10.567814°E)
	2	*S. multistriatus*	Insect	Chiusdino, Italy (43.163653°N, 11.088422°E)
	1	*S. multistriatus*	Insect	Bagno a Ripoli, Italy (43.734871°N, 11.324844°E)
	1	*S. multistriatus*	Insect	Florence, Italy (43.772402°N, 11.176578°E)
	1	*S. multistriatus*	Insect	Vaglia, Italy (43.890112°N, 11.339246°E)

Total DNA from each adult *S. multistriatus* beetle collected from flickering traps, as well as in mother and larval galleries, was extracted singly or in batches of four when it came to the beetles collected in the multi-funnel trap. No surface sterilisation was carried out. Beetles were ground by using Mixer Mill 300 (Qiagen) and DNA from the insect's body was extracted by using the E.Z.N.A. Insect DNA Minikit (Omega Bio-tek), following the manufacturer's instructions.

Total DNA was checked by agarose gel electrophoresis and was quantified using the Nanodrop ND-1000 spectrophotometer (NanoDrop Technologies). The quality of DNA extracted from elm woody tissue was checked using a SYBR-Green real-time PCR endogenous control for the actin gene, following Pepori et al. (2019).

TaqMan MGB probes and primer design

Two sets of primers and TaqMan minor groove binding (MGB) probes were newly designed to obtain genus-specific *Geosmithia* and species-specific *Ophiostoma novo-ulmi* qPCR markers.

The recently-described *G. funiculosa* (Crous et al. 2022) is associated with a broad spectrum of bark beetle species that feed on coniferous and deciduous host plants, including elms and it is phylogenetically close to other *Geosmithia* species found on elm (Pepori et al. 2015; Crous et al. 2022). *Ophiostoma novo-ulmi* ssp. *americana* and ssp. *novo-ulmi* do not differ at the chosen ITS1 target region. These features made these isolates suitable for their use as standard strains for qPCR assay validation.

Primer and TaqMan MGB probes were designed using Primer Express Software 3.0 (Applied Biosystems Foster City, CA, USA), on the basis of the internal transcribed spacer (ITS2) region of *Geosmithia funiculosa* (accession n. KR229885 – isolate CNR28) and ITS1 region for *O. novo-ulmi* ssp. *americana* (accession n. EF429091 – isolate 182E). The TaqMan MGB probes were labelled with the reporter dyes 6-carboxyfluorescein (FAM) and VIC at the 5' end and a minor groove binder non-fluorescent quencher (MGBNFQ) at the 3' end. Primers and probes sequences were reported in Table 3.

Homology of the amplicon sequence (both for *Geosmithia* spp. and *Ophiostoma novo-ulmi*) with the sequences of other species in the NCBI database was performed using standard nucleotide BLAST (BLASTn) (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Primers were synthesised by Eurofins Genomics (Ebersberg, Germany) and probes by Applied Biosystems (Foster City, CA, USA). Specificity of the primers and probes was also tested by qPCR on DNA from axenic cultures (Table 1), as reported below.

Duplex qPCR assay

Real-time PCR was assayed in MicroAmp Fast 96-well Reaction Plates (0.1 ml) closed with optical adhesive and using the StepOnePlus Real-Time PCR System (Applied Biosystems, Life Science, Foster City, CA, USA). Singleplex and duplex qPCR mixtures and thermocycler conditions were tested in this study (data not shown) in order to determine optimal qPCR conditions for the two target pathogens, which were finally set up as follows.

Duplex qPCR was performed in a 25 µl final volume containing: 12.5 µl TaqMan Universal Master Mix (Applied Biosystems,), 300 nM each forward primer (OphF and GeoF), 300 nM each reverse primer (OphR and GeoR), 200 nM each TaqMan MGB probe (OphPr and GeoPr) and 5µl genomic DNA. Each DNA sample was assayed in three replicates. Three wells, each containing 5 µl of sterile water, were used as the no-template control (NTC). For singleplex qPCR assay, only one primer set and one TaqMan MGB probe were used and sterile ddH_2O was added to reach the final volume (25 µl). The PCR protocol was 50 °C (2 min), 95 °C (10 min), 45 cycles of 95 °C (30 s) and 60 °C (1 min).

Data results were analysed using the software SDS 1.9 Sequence Detection System (Applied Biosystems) after manual adjustment of the baseline and fluorescence threshold.

Table 3. Primer and TaqMan MGB probes used in the duplex qPCR assay.

Target	Primers and probes	Sequences (5'-3')[a]	Amplicon length (bp)	Tm (°C)[b]
Ophiostoma novo-ulmi	OphF (Forward primer)	GCCGCCCGAACCTTTT	60	58 58 68
	OphR (Reverse primer)	TGGCTGTTTTTGTTTGTTTCTCA		
	OphPr (TaqMan MGB probe)	**VIC**-AAACCAGTAACGAAACGT-**MGBNFQ**		
Geosmithia spp.	GeoF (Forward primer)	CGCCGTAAAACCCCAACTT	61	59 58 69
	GeoR (Reverse primer)	GTTCAGCGGGTATTCCTACTTGA		
	GeoPr (TaqMan MGB probe)	**FAM**-ACCAAGGTTGACCTCG-**MGBNFQ**		

[a]VIC=fluorescent label (Applied Biosystems); MGBNFQ= minor groove binder non-fluorescent quencher; FAM= reporter dyes 6-carboxyfluorescein. [b]T_m= melting temperature.

qPCR specificity and sensitivity assay and standard curve

The specificity of primers and probes (genus-specific for *Geosmithia* and species-specific for *Ophiostoma novo-ulmi*) were tested both in singleplex and duplex qPCRs using DNA (at final concentration of 5 ng μl^{-1}) from axenic cultures of other strains and species of the target organisms, as well as of closely-related species associated with elm and ubiquitous species (Table 1).

The standard curve was generated using DNA from strain CNR28 (*G. funiculosa*) and strain 182E (*O. novo-ulmi* ssp. *americana*) as standards. For each target species, standard points (ranging from 5 ng μl^{-1} to 2 fg μl^{-1}) were made using ten 1:5 serial dilutions of standard DNA of both target fungi. Each standard curve was built with standards run in both singleplex and duplex qPCR. The minimum amount of template DNA (limit of detection, LOD) that yielded 100% positive results with the singleplex and duplex assay (qPCR sensitivity) was determined. Three replicates of each dilution were analysed and reactions were repeated at least twice. Quantification of both fungal species DNA in unknown samples was made by interpolation from standard curves generated with *O. novo-ulmi* and *G. funiculosa* DNA standards that were amplified in the same PCR run. Reproducibility of the qPCR assay was assessed by computing the coefficient of variation (CV) amongst the mean values in eight independent assays. PCR efficiency was calculated against the slope of the standard curve (Eff = $10^{-1/slope}$ -1) (Bustin et al. 2009), from eight independent experiments.

Validation of qPCR assay in plant tissues

To evaluate the possible interference of plant DNA extract in the newly-designed qPCR assay, the same ten 1:5 serial dilutions (ranging from 5 ng μl^{-1} to 2 fg μl^{-1}) of fungal DNA (*O. novo-ulmi* or *Geosmithia* spp.) were mixed with DNA extracted from healthy elm woody tissue (at 20 ng/tube final concentration) and run on the same qPCR plate of the standard curve (fungal DNA diluted in sterile ddH_2O). All samples were run in triplicate as previously described.

Linearity and sensitivity of qPCR on DNA from ascospore serial dilution

To test the linearity and the sensitivity of each qPCR TaqMan protocol, two different ascospore serial dilutions were obtained from mycelium of axenic culture of *Ophiostoma novo-ulmi* (strain 182E) and *Geosmithia funiculosa* (strain CNR28). Fungal isolates were grown on MEA media and, after five days, the presence of the ascospores was observed using a Zeiss Axioskop 50 optical microscope. Each ascospore suspension was obtained by scraping the surface of mycelium with a sterile scalpel and then placing it in 1 ml of sterile water. The number of ascospores per ml was determined in a Burker Chamber and, for each pathogen, six 1:10 serial dilutions (1:1 *O. novo-ulmi* 1.3×10^7 ascospores per ml; 1:1 *G. funiculosa* 5.6×10^6 ascospores per ml) were prepared. All suspension dilutions were centrifuged for 3 min at 12,000 rpm, the excess water was removed and the ascospore pellets were ground in a 1.5-ml Eppendorf tube using a micropestle (Eppendorf, Hamburg, Germany) in 500 µl of lysis buffer AP1 (EZNA Plant DNA, Omega Bio-tek) and DNA extraction continued with the recommended protocol provided by EZNA Plant DNA kit (Omega Bio-tek, Inc). For each ascospore dilution, 2.5 µl of extracted DNA was assayed using the StepOnePlus Real-Time PCR System (Applied Biosystems) as previously described.

Statistical analysis

For each fungal pathogen (*Ophiostoma novo-ulmi* and *Geosmithia* spp.), pairwise comparison of Cq values of standard points was conducted between duplex and singleplex using the chi-square ($\chi 2$) test. The Bland-Altman plot was used to determinate the agreement between the two assays (Bland and Altman 1986, 2007). The amount of fungal DNA in insects' bodies and elm tissues was expressed as pg fungal DNA/µg total DNA extracted. Differences in *Geosmithia* spp. and *O. novo-ulmi* DNA were detected by the analysis of variance (ANOVA), followed by Tukey's HSD post-hoc test. The significance was evaluated at the 0.05 p-level. Statistical analysis was carried out using XLSTAT (Addinsoft New York, USA).

Results

Specificity and sensitivity of qPCR assays

BLAST search in NCBI showed 95–100% homology between the designed amplicon sequences and the sequence of *Geosmithia* species and *Ophiostoma novo-ulmi* deposited in GenBank.

All DNA from *Geosmithia* spp. isolates (Table 1) were positively amplified after qPCR, using the *Geosmithia*-genus-specific assay. The *Geosmithia* genus-specific assay did not generate any amplicon with DNA from any of the other species tested, such as *O. quercus*, *O. ulmi*, *O. novo-ulmi*, nor with *Epiccoccum* spp. and *Cladosporium* spp.

Ophiostoma novo-ulmi-specific assay successfully amplified DNA from all the *O. novo-ulmi* strains and it did not generate any amplicon DNA with other *Ophiostoma* species tested, including *O. ulmi*, *Geosmithia* spp. or any of other fungal species tested (Table 1). No differences in terms of specificity between singleplex and duplex were observed for the tested isolates.

The standard curves generated with the singleplex and duplex assays did not significantly differ for *Geosmithia* spp. (χ^2 = 0.612; df = 1; P = 0.43) or for *O. novo-ulmi* (χ^2 = 0.167; df = 1; P = 0.68) (Fig. 1). The high level of agreement between singleplex and duplex platforms was confirmed by Bland-Altman plots (Fig. 1). In general, similar levels of agreement between singleplex and duplex for each target gene were reported, with most C_q differences in each comparison falling within the limits of agreements.

The amplification efficiency of duplex qPCR assay was calculated from the slope value of the standard curves according to the equation previously described (Kubista et al. 2006). The slopes of the standard curves were 3.522 for *O. novo-ulmi* and 3.507 for *Geosmithia* spp. and these values corresponded to amplification efficiencies ranging from 92.3% to 92.8% (Table 4). The correlation coefficient (r^2) was 1 and 0.998 for *O. novo-ulmi* and *Geosmithia* spp., respectively, indicating a strong linear relationship between the Cq value and the logarithm of the fungal DNA concentration (Table 4).

The limit of detection (LOD) of both duplex and singleplex qPCR assays were as low as 2 fg μl^{-1} for both *Geosmithia* spp. and *O. novo-ulmi*.

The duplex assay revealed no amplification difference between pure fungal DNA (*Geosmithia* spp. or *O. novo-ulmi*) in sterile water and the same amounts diluted in a mixture containing DNAs of different organisms (*Geosmithia* spp., *O. novo-ulmi* and DNAs from elm wood and insect).

Table 4. Efficiency, linear correlation and assay precision of duplex qPCR assay for the detection of *Geosmithia* spp. and *O. novo-ulmi*.

Fungi and variability experiment	Efficiency (%)	Linear correlation (R^2)	Coefficient of variation %
***Geosmithia* spp.**			
Intra assay	95.3	0.999	1.18 ± 0.13
Inter assay	92.8	0.999	1.3 ± 1.07
Ophiostoma novo-ulmi			
Intra assay	96.8	0.999	1.19 ± 0.01
Inter assay	92.3	0.999	1.06 ± 0.66

Duplex real-time qPCR from plant tissues and bark beetles

All DNA samples were analysed by duplex qPCR for the quantification of *Geosmithia* spp. and *O. novo-ulmi*. No DNA of *Geosmithia* spp. or *O. novo-ulmi* was detected in any of the healthy elm samples analysed. Elm samples with recent or previous seasons' infections showed the exclusive presence of *O. novo-ulmi*, with increasing amounts of the pathogen according to the stage of infection (from 18 pg DNA/µg total DNA in recent infections to 140 pg DNA/µg total DNA in older infections) (Fig. 2).

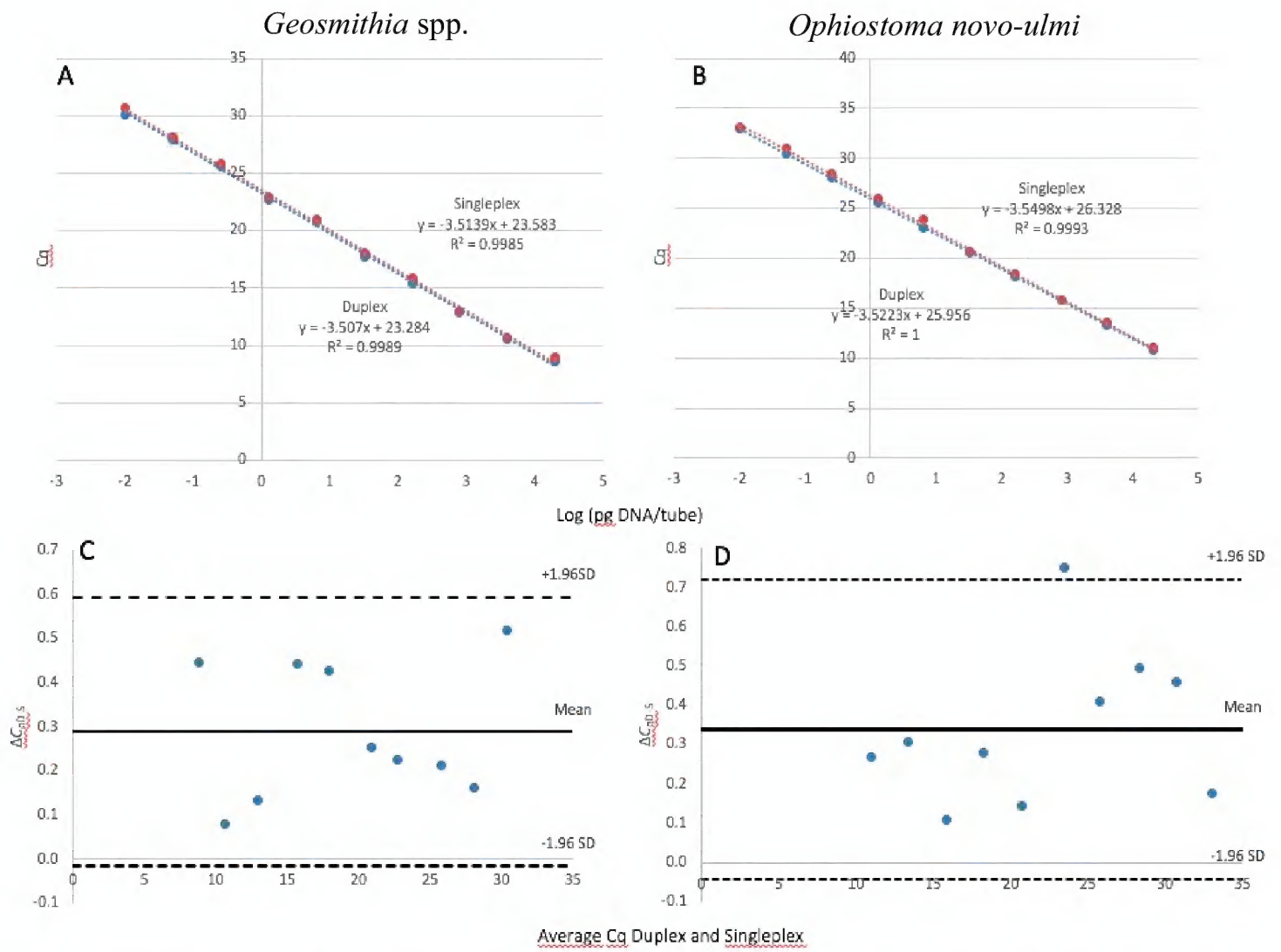


Figure 1. Comparison between singleplex and duplex qPCR **A** standard curve of *Geosmithia* spp. and **B** *Ophiostoma novo-ulmi* generated with the singleplex (blue dots) and duplex (red dots). For each targeted gene, ten different 1:5 serial dilutions (ranging from 5 ng μl^{-1} to 2 fg μl^{-1}) of *Geosmithia* spp. and *O. novo-ulmi* standard DNA were assayed in triplicate. Standard curves were generated by plotting the threshold quantification cycle value (C_q value) versus the logarithmic genomic DNA concentration of each dilution series. The Bland-Altman plot for *Geosmithia* spp. (**C**) and *O. novo-ulmi* (**D**) are shown for the same serial dilutions. The C_q difference between the two methods ($\Delta C_{qD\text{-}S}$) is plotted against the average of both methods (x-axis) for every individual pair of measurements. The interval of the mean of the difference ± 1.96 times the standard deviation (SD) defines the 95% interval of the limits of agreement.

Duplex qPCR results revealed the presence of both fungi in all EBB samples, collected in different stages of their biological cycle (including samples from frass collected in the galleries). In particular, significantly higher quantities of *Geosmithia* spp. DNA compared to *O. novo-ulmi* were found on female EBB collected after ovideposition ($p < 0.0001$, Fig. 2A), corresponding to 63% of the amount of *Geosmithia* found inside the insect galleries (Fig. 2B). The presence of *Ophiostoma* detected was significantly lower ($p = 0.05$) than *Geosmithia* in all EBB samples analysed, especially in the insects present in the galleries (Figs 2, 3). The quantity of *Geosmithia* DNA in wood frass and callow adult insects was significantly higher than in pupae and larvae ($p < 0.0001$; Fig. 2A).

A

	Source	N° of Samples	Mean of fungal DNA (pg DNA/µg total DNA) (±SEM)	
			O. novo-ulmi	*Geosmithia* spp.
***Ulmus* spp.**	Healthy	14	0	0
	New DED infection	14	18 (±2)	0
	Old DED infection	26	140 (±38)	0
	Frass from EBB galleries	12	0.28 (±0.4)	356 (±108)
Scolytus multistriatus	Larvae	10	0.1 (±0.01)	1.5 (±0.47)
	Pupae	8	0.2 (±0.1)	2.2 (±0.7)
	Adult in the galleries	9	1.14 (±1)	636 (±137)
	Callow adult	30	0.3 (±0.16)	23 (±4.6)

B

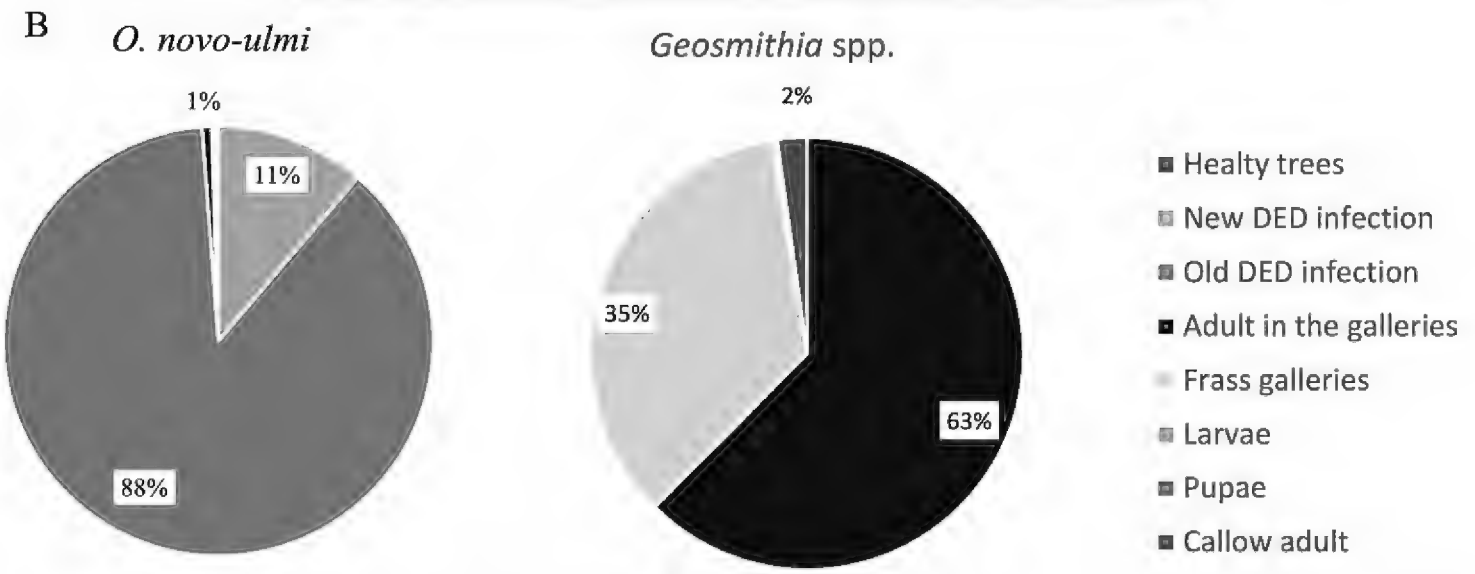


Figure 2. Fungal DNA of *O. novo-ulmi* and *Geosmithia* spp. on analysed samples by using duplex qPCR assay **A** Mean of fungal DNA (pg DNA/µg total DNA) ± SEM (Standard Error of the Mean) **B** percentage presence of *O. novo-ulmi* and *Geosmithia* spp. DNA in plant tissues and EBB samples analysed.

Linearity and sensitivity of qPCR on DNA from ascospore serial dilution

DNA extracted from ascospore serial dilution showed a linear relationship for *O. novo-ulmi* ($R^2 = 0.999$) and *Geosmithia* spp. ($R^2 = 0.999$) (Fig. 4). Fungal DNA quantification for *O. novo-ulmi* ranged from 31 pg μl^{-1} to 10 fg μl^{-1} corresponding to 10^7 to 10^2 ascospore/ml; while for *Geosmithia* spp. from 5.8 pg μl^{-1} to 3 fg μl^{-1} corresponding to 10^6 to 10 ascospore/ml.

Discussion

Dutch Elm Disease is still causing massive damage in Europe and the death of elms is still catastrophic in ecological and economical terms through the loss of genetic diversity and trees lost from urban and natural forest stands (Santini and Faccoli 2015).

The detection of fungi by traditional methods, such as isolation from plant tissues and insect bodies, may be sometimes challenging and time-consuming, seriously

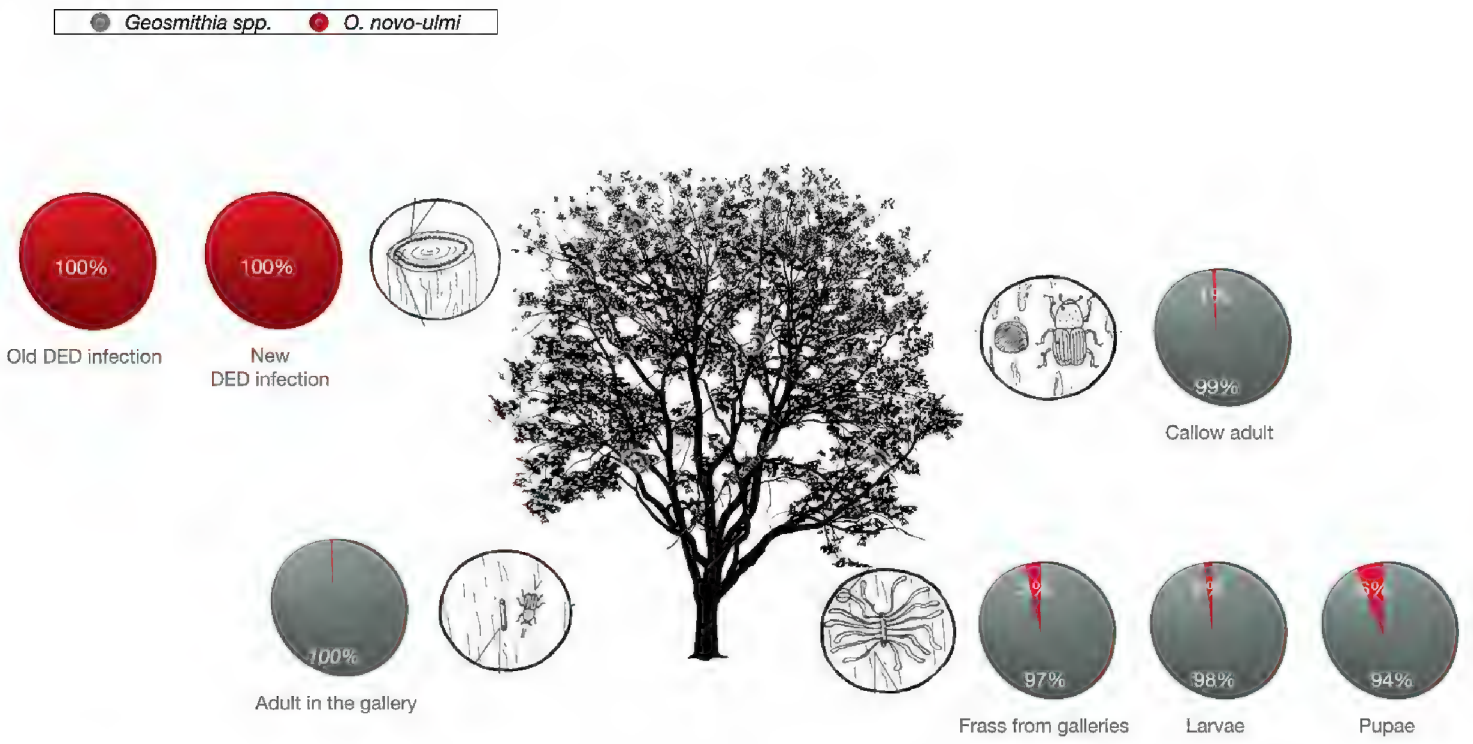


Figure 3. Proportion of target DNAs (%) at different DED infection stages.

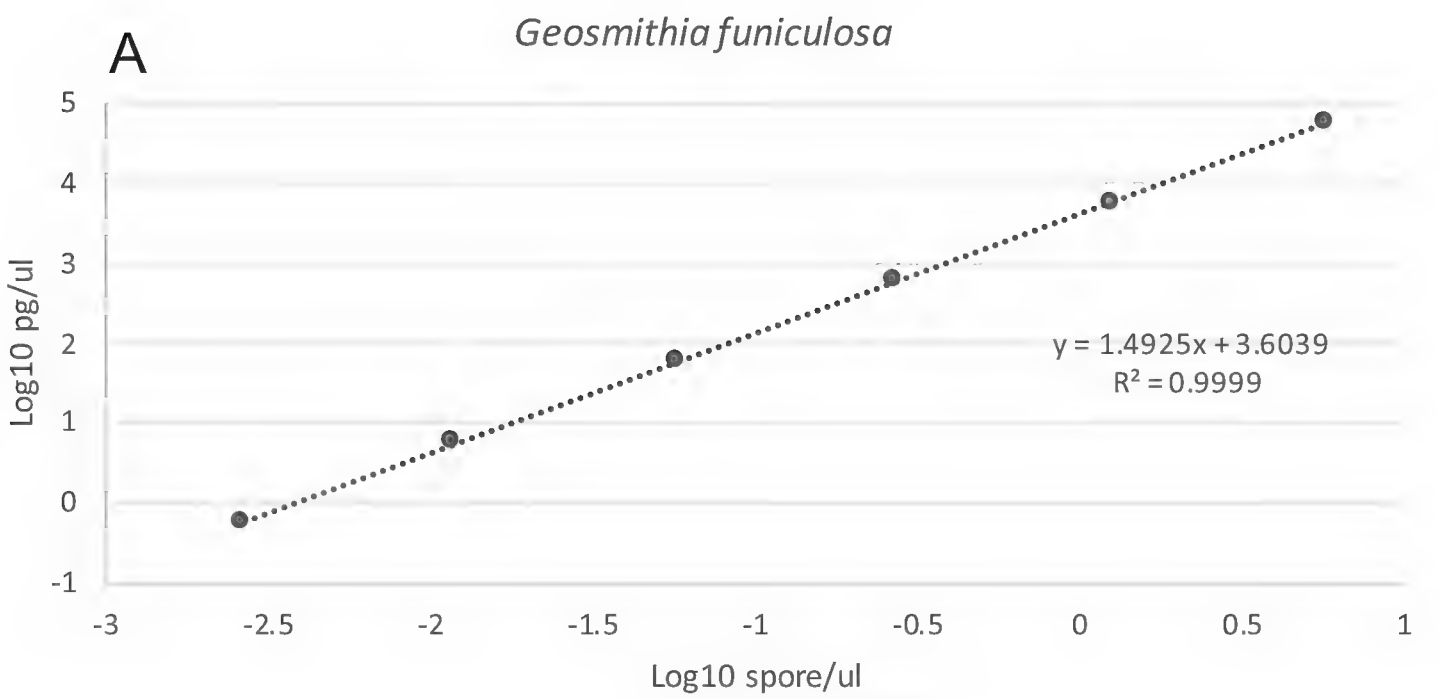
impairing our knowledge of their biological cycles. In addition, these methods do not allow quantification of the target organism. DNA sequence-based molecular tools, such as real-time PCR, digital PCR or, even if indirectly, LAMP (Hardinge and Murray 2020) and HTS, are increasingly used to enable accurate and specific detection and quantification from any substrate (Lindahl et al. 2013).

Multiplex qPCR is an increasingly utilised method (Bonants and te Witt 2017; Luchi et al. 2018; Rizzo et al. 2020) allowing simultaneous detection of different microorganisms in the same reaction, thus significantly reducing both the quantity of samples and the overall cost of the analysis. The use of a multiplex assay may prove particularly important to distinguish pathogens that cause similar symptoms, as in the case of *Fusarium circinatum* and *Caliciopsis pinea*, which cause comparable symptoms on *Pinus radiata* (Luchi et al. 2018) or the study of the four European species of *Heterobasidion* that attack conifers (Ioos et al. 2019).

In this study, the developed and validated duplex qPCR assay was able to detect and quantify the presence of *Geosmithia* spp. and *O. novo-ulmi* from different matrices (frass and plant tissue; adults, larvae and pupae of bark beetles) collected from healthy and DED-symptomatic elms.

This duplex qPCR assay showed high reproducibility and specificity for both genus-specific *Geosmithia* spp. and species-specific *O. novo-ulmi* and high sensitivity (LODs 2 fg μl^{-1}, for both fungi). This assay allowed the detection in elm trees of *O. novo-ulmi* infections before symptoms had fully developed, as well as the presence of *Geosmithia* spp. in different host tissues and on the insect body.

Our results confirm that *Geosmithia* is closely associated to EBB galleries, as also reported by Kolařík et al. (2008), showing extremely high amounts on the EBB female bodies and in maternal gallery frass.



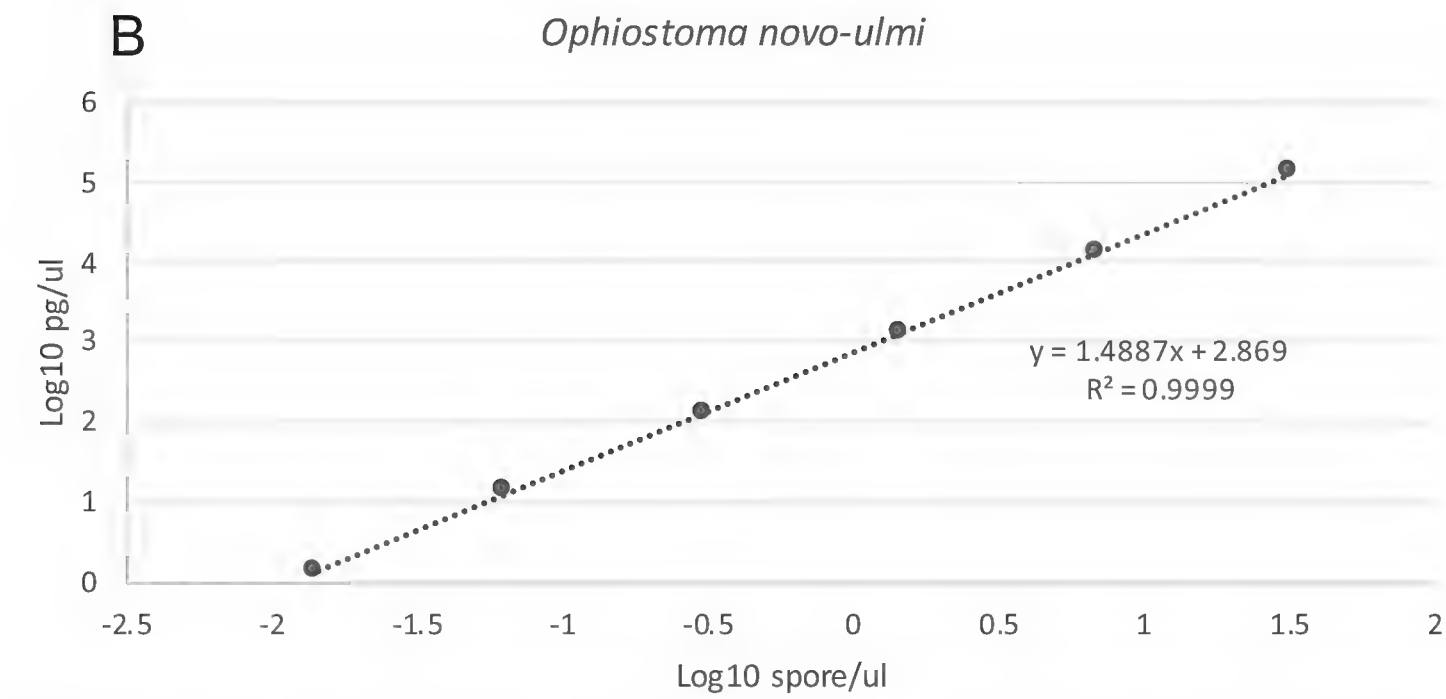


Figure 4. The quantification of **A** *Geosmithia funiculosa* and **B** *Ophiostoma novo-ulmi* extracted from ascospore dilutions. For each sample, dilution data were reported as the median value of triplicates ±SD.

Our observations indicate that the humidity and temperature conditions within the subcortical galleries seem to promote the fitness of the fungi studied here, particularly *Geosmithia*. In addition, the results show that *Geosmithia* is always present in beetle galleries along the studied period, but the detected DNA quantity decreases significantly as the insect's maturation progresses, i.e. from the time of ovideposition until the callow adults flicker.

This study confirms the association between bark beetles and *Geosmithia*, as also reported by other studies (Kolařík et al. 2008, 2017; Pepori et al. 2018; Huang et al. 2019) and highlights that this association is constant throughout the life of the bark

beetle and is not only specific to the subcortical developmental stage. Moreover, fungi benefit from this association because the beetles transport them to new host plants (Paine et al. 1997; Six 2003; Six and Wingfield 2011) and prepare a suitable habitat for their growth. In the galleries dug by insects, the fungi become metabolically more active because they have access to a constant supply of nutrients such as decaying wood (Stokland et al. 2012).

The elm bark beetles are generally unable to digest the lignin, cellulose and hemicellulose components that make up xylem tissues (Dadd 1970; Geib et al. 2008) and feed primarily on the phloem. However, for some phloem-feeding beetles, phloem tissues remain relatively low in usable nitrogen and sterols and, thus, the associated fungi can serve as a complementary source of nutrients (Six 2012). It has been observed that symbiotic fungi are able to access nitrogen stored in the sapwood and translocate it into the phloem where the larvae and pupae of bark beetle feed (Stokland et al. 2012). Bark beetles and ambrosia beetles, as reported also by Kolařík and Kirkendall (2010) and Veselská and Kolařík (2015), use these fungi as principal nutritional symbionts and recently new *Geosmithia* species associated with ambrosia beetles have been described in a tropical forest in Costa Rica (Kolařík and Kirkendall 2010).

EBBs are the main vectors of *Geosmithia* spores on their body and maybe use the fungus as a complement to their nutrition, especially during the larval and pupal stages of their life cycle that takes place within the galleries under the elm bark. However, more studies are needed to confirm this hypothesis.

The callow adults complete their maturation over a few days by digging short feeding burrows in the phloem of the twig and sapwood of healthy elms (Fransen 1939; Webber and Brasier 1984), where they deposit the DED fungal spores. This study shows that these insects carry large quantities of *Geosmithia* and much less of *O. novo-ulmi* (Fig. 3). The spores of the latter reach the xylem and move in the vessels through a phase of yeast multiplication (Webber and Brasier 1984), giving rise to the infection process. *Geosmithia*, at least in this first phase, is not detectable and this could mean that it does not find optimal conditions to spread or it is translocated in other parts of the plant. In fact, although the new insects flicker from the bark of dying elms carrying 99% *Geosmithia* spores, to the xylem of elm trees experiencing new attacks, we found only the presence of the DED pathogen. These results are in contrast with those reported by Pepori et al. (2018), who found that the artificial inoculation of both fungi in the same elm clone resulted in significantly lower symptoms than single inoculations of *O. novo-ulmi*. Maybe the reason lies in the fact that artificial inoculations, generally performed in the internodal section of the twig, circumvent the natural plant reaction, while beetles dig their burrow at the twig crotches (Santini and Faccoli 2015).

None of the target DNAs was detected in healthy elm tissues and only *O. novo-ulmi* DNA was detected in DED-symptomatic plants, confirming that *Geosmithia* does not adapt to the conditions of living plants tissues or even in xylem of plants with early DED symptoms (Pepori et al. 2018).

These findings show that this fungus is not an endophyte, at least in elm. Instead, *Geosmithia* was detected in abundance on EBB bodies and in EBB tunnels in decaying plants. Our analyses suggest that the presence of this fungus is mostly associated with

the breeding activity of the vector insect on elm trees as already observed in other studies (Kolařík et al. 2007, 2008; Kolařík and Jankowiak 2013).

In conclusion, the duplex qPCR technique developed in this work is extremely sensitive and able to specifically identify and quantify the presence of both *O. novo-ulmi* and *Geosmithia* spp. in plants with different levels of DED symptoms, on EBB larvae, pupae and wood frass from maternal and larval galleries and on the body of callow adult insects, providing better insight into the dynamics of this complex fungus-fungus association mediated by *S. multistriatus*. This work provided solid data on the actual DNA quantity of the two fungi at the different steps of the DED cycle, thus gaining a better understanding of the role and interactions occurring amongst all the pathosystem players.

Dutch elm disease continues to be extremely damaging on planted and natural elm stands in Europe. Critical thresholds comparable to those that led to the decline of the first epidemic do not appear to have been reached and the current disease dynamic seems likely to continue.

Moreover, an increasingly warming climate could have a great influence on beetle epidemics, their aggression, population dynamics and migration (Bentz and Jönsson 2015), allowing the expansion of the DED epidemic to more northern latitudes (Jürisoo et al. 2019, 2021).

Several aspects of *O. novo-ulmi-Geosmithia-Scolytus* interactions within the DED pathosystem need to be further studied and more in-depth information on the biological cycle of *Geosmithia* spp. during the flickering period of new generations will be essential to use this fungus as a biocontrol agent of DED and finally allow European elms to re-populate the landscape.

Acknowledgements

This study was supported by the HOMED project (http://homed-project.eu/), which received funding from the European Union's Horizon 2020 Research and Innovation Programme under grant agreement No. 771271. We thank Dr. Miroslav Kolařík for providing *Geosmithia* spp. isolates from Czech Republic, Croatia and Spain. Authors wish to warmly thank Dr. Matthew Duncan Haworth for English language editing. Authors wish to thank the anonymous referees who helped to significantly improve the manuscript.

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